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Perspective

A Novel Role for Hedgehog in T-Cell Receptor Signaling

Implications for Development and Immunity

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ABSTRACT

The Hedgehog (Hh) signaling pathway is a key regulator of both embryonic development and homeostasis of adult tissues, including thymus and blood. In the thymus, Hh signals for differentiation, survival and proliferation in the early stages of T cell development, before TCR gene rearrangement. Our recent data has shown that Hh signaling also modulates T cell receptor (TCR) signal strength in more mature T lineage cells. We showed that constitutive activation of the Hh pathway in thymocytes (by transgenic expression of the transcriptional activator form of Gli2) decreased TCR signal strength with profound consequences for the thymus—allowing self-reactive T cells to escape deletion and altering T cell CD4/CD8 lineage decisions. In contrast, in the Sonic Hh deficient thymus, TCR signaling was increased, again influencing both TCR repertoire selection and CD4/8 lineage commitment. In peripheral T cells, the transcriptional changes induced by activation of the Hh signaling pathway lead to reduced T cell activation. Hh signaling also attenuated ERK phosphorylation and proliferation in mature T cells on TCR ligation. Modulation of TCR signal strength by Hh pathway activation has importance for immunity as the presence or absence of Hh in the environment in which a T cell is activated would shape the immune response.

INTRODUCTION

The hedgehog (Hh) family of secreted intercellular signaling molecules (Sonic, Indian and Desert Hh) modulate target gene expression via the Gli transcription factors. Hh proteins are essential morphogens during embryogenesis and the homeostasis of adult tissues,[1-4] and can influence differentiation, cell cycle progression and survival.[1,5] In a classical model, a morphogen specifies cell fate by establishment of a morphogen gradient, in which its movement from a polarised source provides positional information for patterning and differentiation in a solid tissue. In addition to its competence to transduce the signal, the response of the receiving cell depends on the concentration and duration of the signal received and therefore on its position in the tissue. In contrast to organogenesis of solid tissues, the immune system is 'fluid', made up of multiple cell types that move through the body and interact, differentiate and respond in many different environments. Despite this apparent difference, Hh signaling has also turned out to be an important regulator of the immune system.[2,6-12]

The Gli transcription factors (Gli1, Gli2 and Gli3) are key to the interpretation of the Hh gradient by the receiving cell.[1,5] They have distinct temporal and tissue-specific expression patterns and functions. Gli2 is necessary to initiate the first transcriptional changes induced by Hh signaling.[13] Gli1 is solely an activator of transcription, but Gli2 and Gli3 can be processed to activate or repress target-gene transcription, according to the presence or absence of Hh respectively.[13-16] Gli functions are affected by their cellular context, and are dependent both on cell type and the Hh environment of that cell.[17,18] The interpretation of the Hh signal in a given cell relies on the balance of activator and repressor forms of Gli proteins produced.

HH SIGNALING IN THE THYMUS

The specialised environment of the thymus supports the maturation of haematopoietic progenitors into functional T lymphocytes and this involves bi-directional signaling between the thymic epithelium and developing thymocytes. During T cell development, thymocytes pass through stages that can be defined by the expression of cell surface markers: $CD4^-CD8^-$ double negative (DN) cells differentiate into $CD4^+CD8^+$ double

positive (DP) thymocytes, which mature to become CD4 single positive (SP) and CD8 SP thymocytes. Signaling by a functional pre-T cell receptor (pre-TCR) is necessary for differentiation from DN to DP cell.[19] Maturation from DP to SP cell requires the expression of a functional, MHC-restricted αβTCR and involves TCR repertoire selection.[20,21] Positive selection of thymocytes that express TCR with appropriate affinity for self-MHC molecules ensures functional self-restriction, while negative selection of thymocytes that express TCR with high affinity for self removes overtly self-reactive T cell clones; therefore cell fate is at least in part determined by the strength of the signal transduced by the TCR.[21] Mature T cells then exit the thymus to populate peripheral lymphoid organs where they can be activated on TCR binding to peptide antigen presented by major histocompatibility complex (MHC) molecules. CD4 T cells recognise antigen as peptide in the context of self MHC Class II molecules, whereas CD8 T cells recognise antigen as peptide in the context of self MHC Class I molecules.

Hh signaling is important in T cell development, in both humans and mice.[2,6,7,9-12] Shh is expressed by thymic epithelial cells and components of the Hh signaling pathway are expressed in the lymphoid and stromal compartments of the thymus.[2,6,9,11,12] Hh signaling is involved in the homeostasis of DN cells, differentiation from DN1 to DN2[6,7,10,11] and the DN to DP transition.[2,7,10,11]

We have recently investigated the function of Hh signaling at later stages of murine thymocyte development. We have shown that Hh signaling is involved in the maturation of immature DP cells to mature SP thymocytes and that it can affect TCR repertoire selection and CD4/8 lineage choice.[12]

HH SIGNALING ALTERS TCR REPERTOIRE SELECTION AND CD4:CD8 RATIO IN DEVELOPING THYMOCYTES

We produced transgenic mice with a transcriptional activator form of the Gli2 protein ($Gli2\Delta N_2$)[14] under the control of the T-lineage-restricted Lck promoter.[22,23] This Lck-$Gli2\Delta N_2$ transgene induces the transcriptional events normally caused by active Hh signaling, allowing us to assess the effect of constitutive activation of the Hh signaling pathway specifically in T cells.

Expression of the Lck-$Gli2\Delta N_2$ transgene decreased the production of CD4 SP cells in the thymus, and reduced the CD4:CD8 ratio. Treatment of wild type (WT) thymus explants with recombinant Shh protein (r-Shh) also decreased the CD4:CD8 ratio, confirming that Hh can influence the CD4/8 lineage decision. Conversely, analysis of $Shh^{-/-}$ thymi showed increased production of CD4 SP cells and the CD4:CD8 ratio, confirming that Hh signaling, and Shh in particular, does affect CD4/8 lineage commitment under physiological conditions (Fig. 1A). Commitment of the DP population to the CD4 or CD8 lineage is complicated by the fact that the mature CD4 and CD8 cells must express TCR restricted by the appropriate MHC molecule (by Class I in the case of CD8 SP cells, and by Class II in the case of CD4 SP cells). Numerous models have been proposed to explain how the DP population commit to the CD4 or CD8 lineage,[24] and TCR signal strength during repertoire selection has been shown to influence SP lineage commitment, with a stronger signal favouring differentiation to CD4 SP.[25-27] The fact that manipulation of the Hh signal received by developing thymocytes influenced the CD4/8 lineage decision therefore raised the possibility that Hh was modulating TCR signal strength, and in this way affecting the lineage decision. Interestingly, we found that cell surface expression of CD5, which correlates with TCR signal strength,[28] was decreased in the Lck-$Gli2\Delta N_2$ transgenic thymocytes, and after treatment of WT thymi with r-Shh. This reduction in cell surface CD5 expression therefore supported the idea that the reduced proportion of CD4 SP cells found in the Lck-$Gli2\Delta N_2$ transgenic, and after treatment of WT thymi with r-Shh, is a result of Hh signaling acting to reduce TCR signal strength.

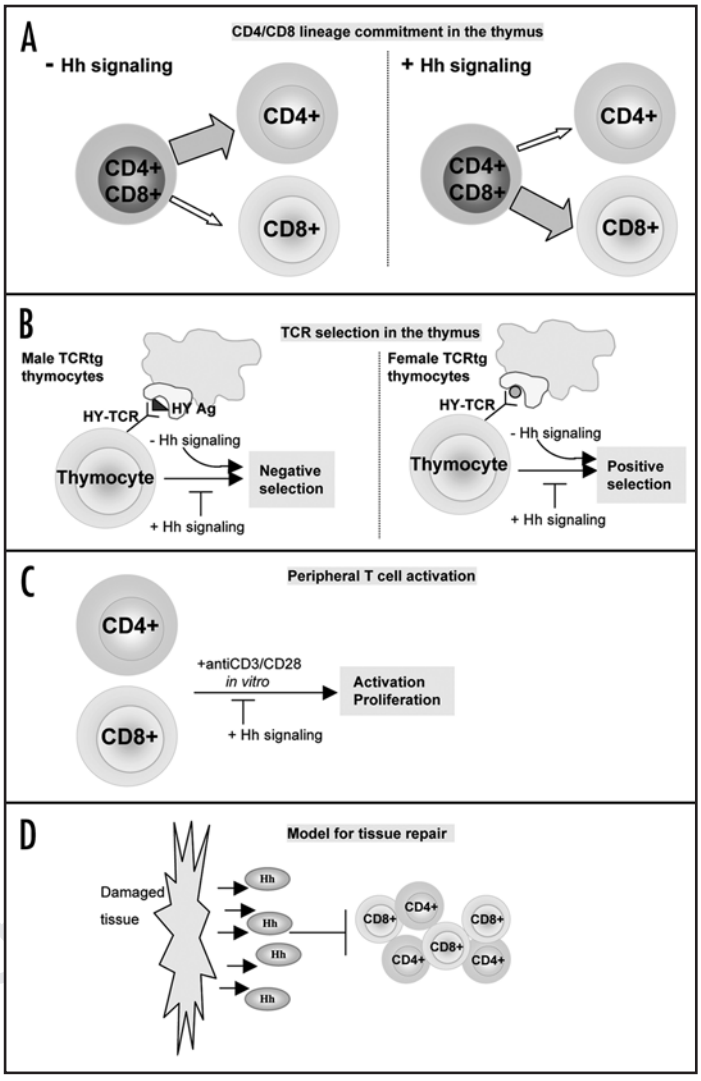

Figure 1. Summary of Hedgehog pathway activity in CD4/8 lineage commitment, TCR repertoire selection and peripheral T cell activation. (A) Reduced Hh signaling (i.e., in $Shh^{-/-}$ thymus) promotes CD4 lineage commitment resulting in increased CD4:CD8 ratio (left). In contrast increased Hh signaling (i.e., in transgenic Lck-$Gli2\Delta N_2$ thymocytes with constitutively active Hh signaling, or in thymus explants treated in vitro with r-Shh) results in reduced CD4:CD8 ratio (right). (B) Hh signaling negatively influences both positive and negative selection. Deletion of CD8 thymocytes expressing the transgenic TCR recognizing the male antigen HY (in the context of MHC class I) is impaired in Lck-$Gli2\Delta N_2$ male thymus but enhanced in $Shh^{-/-}$ male thymus (left). Positive selection of Lck-$Gli2\Delta N_2$ CD8 cells expressing the HY-TCR is also negatively affected in the female thymus whereas positive selection of HY-TCR transgenic thymocytes is promoted in $Shh^{-/-}$ female thymus. (C) Hedgehog signaling antagonizes in vitro T cell activation. Proliferation and activation of peripheral Lck-$Gli2\Delta N_2$ T cells was inhibited. (D) Proposed model for possible role of Hh signaling and T cell activation during tissue repair or tumor development. Damaged tissues or tumors might produce and secrete Hh proteins that can signal to lymphoid cells present. We predict that Hh pathway signaling would limit T cell activation in response to Hh secretion by damaged tissues and tumors.

To test this hypothesis, we used the HY-TCR transgenic model that allowed the study of positive and negative selection of CD8 SP cells.[29] HY-TCR transgenic T cells express a TCR that recognises a male antigen, restricted by a Class I MHC molecule. In male HY mice, negative selection is highly efficient with deletion of the male-reactive CD8 and DP population.[29] When we added the Lck-Gli2ΔN_2 transgene to male mice positive for the HY-TCR we found a small population of HY-TCR positive (auto-reactive) CD8 SP cells that had escaped deletion.[12] The HY-TCR model can also be used to assess positive selection to the CD8 lineage in female mice.[29] In HY-TCR females with the Lck-Gli2ΔN_2 transgene, we observed reduced positive selection of thymocytes expressing the transgenic TCR to the CD8 SP lineage (Fig. 1B).

To confirm the physiological significance of this observation we analysed Shh deficient HY-TCR mice. Again, analysis of Shh deficiency showed the opposite effect to the Lck-Gli2ΔN_2 transgenic, with more efficient deletion in males and increased positive selection in females.[12] The altered selection observed using the HY-TCR model thus showed that Shh signaling does act to reduce TCR signal strength in developing thymocytes during TCR repertoire selection (Fig. 1B).

Shh is secreted by thymus epithelial cells scattered in the medulla and at the corticomedullary junction,[2,6,9] where TCR repertoire selection takes place.[30] Proximity to the Shh source would dictate the strength of TCR signal received by a given cell, allowing the architecture of the thymus to influence the T cell repertoire by setting up a Hh gradient. As developing thymocytes move through the thymus they will encounter different strengths of Shh and therefore the outcome of TCR-ligation for positive and negative selection and CD4/CD8 lineage decisions will be dependent on their position in the thymus relative to the Shh signal. Not all medullary epithelial cells express Shh,[2,6,9] so it is likely that Shh-secreting-epithelial cells are specialised for particular functions, setting up 'niches' to promote positive selection and commitment to the CD8 lineage.

The way in which the transcriptional changes induced by Hh pathway activation modulate the TCR signal during repertoire selection are unknown, and further investigation is required.

HH SIGNALING ATTENUATES PERIPHERAL T-CELL ACTIVATION AND PROLIFERATION

On antigen recognition in the periphery, naïve T cells are stimulated to clonally expand and differentiate into effector cells. This requires costimulation from antigen presenting cells (APC) and outcome is influenced by cytokines, the microenvironment and the strength of the TCR signal received.[31]

Peripheral T cell activation and proliferation require two distinct signaling events. The TCR-CD3 complex recognises antigen presented as peptide bound to MHC molecules on the surface of APC, and signals for T cell activation. In addition, for full T cell activation a costimulatory signal is required, transduced by binding of CD28 on the T cell to CD80/CD86 on the APC. These events at the cell surface trigger intracellular signaling: Immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3 polypeptides are phosphorylated by Lck causing recruitment and activation of ZAP-70. This results in the activation of multiple signaling cascades including the MAPKinase, PKC, DAG Kinase, PI3Kinase and Calcineurin pathways, leading to the activation of transcription factors such as NFκB, NFAT and AP-1. These transcription factors bind to and activate transcription of genes necessary for T cell activation, including *IL-2*.[32-34] Secretion of IL-2, a potent T cell growth factor, and upregulation of IL-2 Receptor expression by the T cell, allow autocrine control of T cell proliferation.

The strength of signal transduced by the naïve T cell on TCR binding, as determined by antigen concentration and affinity and duration of the interaction, influences further T cell maturation to specialised effector populations.[35] The interaction between the T cell and the APC is enhanced by adhesion molecules and integrins. These are differentially expressed depending on the activation and differentiation status of the cell, and this is itself regulated by cytokines and signals from the environment.[36] The final outcome of the activating signals is therefore additionally dependent on the microenvironment of both the T cell and the APC. One possible influence on T cell activation could be Hh secretion at the site of activation. The role of Shh in peripheral T cell activation and function, however, is controversial.[6,37-39] We therefore used the Lck-Gli2ΔN_2 transgenic model to investigate the effect of Hh pathway activation on T cell activation.

In these experiments, in vitro activation of Lck-Gli2ΔN_2 transgenic T cells by ligation of TCR and CD28, attenuated T cell activation, indicating that the Hh signaling pathway negatively affects TCR-induced T cell activation.[12] The presence of the Lck-Gli2ΔN_2 transgene also seriously affected the ability of T cells to proliferate in response to TCR and CD28 ligation[12] (Fig. 1C).

Hh signaling has long been linked to proliferation, with an increase in Hh signaling generally found to enhance proliferation (for example in the development of limb, taste buds, neural crest and thymus).[4,6,10,40-44] Consistent with Hh promoting cell expansion, the first Gli protein to be discovered, Gli1, was initially identified as an amplified gene and potential oncogene in a human glioma line,[45,46] and aberrant Hh signaling has been implicated in many common malignant tumors such as small cell lung carcinoma, pancreatic, stomach and prostate cancer.[47-50]

By analysis of loss-of-function mutants, T lineage studies have shown that Hh signaling promotes proliferation of early DN thymocytes.[6,10] In contrast, we have recently found that constitutive activation of the Hh pathway inhibits the proliferation of mature T cells.[12] The effect of Hh signaling on proliferation has also appeared ambiguous in other systems, in particular in the retina.[51] In chick and mouse retina Shh activation resulted in increased BrdU incorporation suggesting that Hh signaling promotes proliferation.[52-54] However, Hh mutant Zebrafish exhibited prolonged retinal proliferation due to the inability of precursor cells to exit the cell cycle.[55] Recent work has suggested that Hh signaling may effect stem, progenitor and mature cells differently, with Hh signaling either promoting proliferation or pushing cells out of the cell cycle resulting in reduced proliferative ability depending on the state of differentiation of the cell.[56] The T cell lineage seems to provide another example of Hh activation promoting proliferation of the progenitor cell, but limiting the proliferation of the more differentiated mature cell. This difference in outcome could reflect differences in the intracellular context in which the Hh signal is transduced, leading to the transcriptional regulation of distinct sets of target genes. Alternatively, the inhibition of TCR-induced mature T cell proliferation could be the result of reduction in TCR signal strength by the pathway, rather than a direct effect on cell cycle regulator target genes. While early thymocyte progenitors must expand rapidly to fill the DP pool, and do not yet express a TCR, in differentiated T cells proliferation must only occur as a consequence of appropriate activation in response to antigen, after stimulation through their TCR. Thus, quite different

processes initiate proliferation, consistent with the opposing effects of Hh signaling in the T lineage, in accordance with maturity.

ERK PHOSPHORYLATION AND HH SIGNALING

To dissect further the effect of Hh signaling on TCR signal strength, peripheral T cell activation and proliferation, we followed the kinetics of MAPkinase pathway activation by measuring ERK phosphorylation after TCR-induced activation by Western blot.[57] Presence of the Lck-Gli2ΔN_2 transgene led to much delayed and reduced levels of ERK phosphorylation, which was further confirmed to be T-cell specific using a FACS-based assay.[12]

This reduced ERK phosphorylation observed in our transgenic T cells is of particular interest, as previous accounts of Hh pathway involvement in other tissues are often linked to improved ERK phosphorylation.[58-61] For example, addition of Shh during retinal regeneration, led to an increase in ERK phosphorylation indirectly via the FGF/FGFR pathway.[59] The mechanism by which the transcriptional events induced by Hh signaling reduced TCR-induced ERK phosphorylation is unknown, however it must be proximal to TCR ligation, as ERK phosphorylation is an immediate consequence of TCR ligation.[62]

The MAPKinase pathway is a key regulator of cell proliferation.[57] ERK activity must be sustained late into G_1 to enable successful S-phase entry[63] and inhibitors of ERK phosphorylation have been shown to inhibit the proliferation of many cell types including T cells.[57] A reduction in ERK phosphorylation leads to a decrease in the transcription of ubiquitous mediators of cell cycle progression and in the case of activating T cells, also of *IL-2*[34,57,64] any of which could lead to the observed attenuation of proliferation in the Lck-Gli2ΔN_2 cells. In our experiments, addition of IL-2 to activated cultures restored proliferation of Lck-Gli2ΔN_2 T cells to WT levels, suggesting that a reduction in *IL-2* gene transcription (caused by attenuated MAPkinase activity[64]) is responsible for the proliferative defect. The fact the Lck-Gli2ΔN_2 T cells can proliferate in response to IL-2 demonstrated that the transcriptional changes induced by active Hh signaling did not inherently inhibit T cell division.

Thus, we favour a model in which the decreased proliferation and ERK activation of mature T cells on TCR ligation, caused by constitutive Hh signaling, is a consequence of reduced TCR signal strength mediated by Hh-dependent transcription, rather than a direct effect of Hh on the cells' proliferation per se.

IMPLICATIONS FOR IMMUNITY AND DISEASE

Modulation of TCR signal strength by Hh has profound implications for immunity. Presence of Hh would alter the threshold required for T cell activation, and therefore determine the outcome of the naïve T cell's interaction with antigen, with consequences for anergy, regulation, tolerance and inflammation. This has significance for immune responses, as localised sources of Hh in the T cell microenvironment could influence the outcome of TCR signaling. In the future, it will be important to test this hypothesis in mouse models and in human disease.

Hh proteins are expressed in skin, gut and lung.[1] These tissues are subject to recurrent immune challenge, as they are the sites of entry of external pathogens from the environment, from food and air. They are also subject to inflammatory and autoimmune diseases. Hh expression in these tissues during renewal or remodelling after infection or tissue-damage could function to dampen down the immune response, protecting against the induction of inflammatory or autoimmune diseases (Fig. 1D). By reducing lymphocyte responses to antigen, Hh signaling may also be involved in immune evasion by tumours. Aberrant Hh signaling is common in many cancers[5,17] and secretion of Hh by a tumour could potentially diminish the immune response to that tumour, allowing escape from immune surveillance.

Changes in Hh signaling could be involved in the aetiology of autoimmune disease and mutations in components of the pathway could increase disease susceptibility, either by allowing self-reactive T cells to escape negative selection in the thymus, or by reducing the TCR signal threshold for mature T cell activation. In the thymus, mutations increasing either Shh secretion by epithelial cells, or signal interpretation by thymocytes, would allow those self-reactive clones that would normally be deleted, to mature and escape into the peripheral lymphoid organs, potentially leading to autoimmune disease. In contrast, mutations leading to decreased Hh signal in peripheral T cells (via decreased secretion in the environment or signal interpretation by the T cell) would increase the TCR signal, potentially leading to inappropriate T cell activation.

The role of Hh signaling in T cells (and immunity) is a new field, so there is still much work to be done to dissect its functions and gain insight into the molecular mechanisms. It will be important to understand how Hh modulates the TCR signal, and to investigate the effect of Hh gradient on the signal. It will also be interesting to test if Hh signaling regulates other components of the immune system.

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